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A novel procedure in combination of genomic sequencing, flow cytometry and routine culturing for confirmation of beer spoilage caused by *Pediococcus damnosus* in viable but nonculturable state

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ABSTRACT

Spoilage bacteria had been shown to form viable but nonculturable (VBNC) state maintaining food spoilage capability. In this study, a novel procedure was used to confirm a beer spoilage case caused by a *Pediococcus damnosus* strain in the VBNC state. Firstly, flow cytometry, routine culturing and PMA-PCR methods were used to identify approximately 10^3 cells/ml VBNC cells in the spoiled beer sample based on the difference between CFU and viable cell numbers. Secondly, genomic sequencing showed all acquired scaffolds were identical to the genome of *P. damnosus* with no existence of other species or isolates. In addition, VBNC cells were obtained in both simulation conditions, including beer low temperature storage and subculturing. MRS agar supplemented with catalase was found to resuscitate VBNC cells. Normal, VBNC and resuscitated cells showed similar level of beer spoilage capability. As concluded, a novel procedure, in combination of genomic sequencing, flow cytometry and routine culturing was used to confirm VBNC cells in spoiled beer sample, providing direct evidence on the beer spoilage caused by VBNC *P. damnosus* cells, and will aid in further study on VBNC state in food industry so that more evidence on food safety problem caused by VBNC microbes will be shown.

1. Introduction

Bacterial cells in viable but nonculturable (VBNC) state has posed a major issue for food quality as VBNC cells yields false negative results during microbiological identification by culturing. The VBNC state has been considered to be a survival strategy of over 80 non-sporeforming bacterial species in response to environmental stress conditions (Yamamoto, 2000). Microorganisms in the VBNC state do not grow in routine culture media, but are still metabolically active and usually capable of resuming growth when certain stress conditions are relieved (Oliver, 2010). Problems posed by VBNC state bacteria are mainly due to the nonculturability in conventional laboratory media, leading to the difficulty in detection, along with the active metabolism (Sachidanandham & Gin, 2009). Interestingly, *in vitro* developed fungal-bacterial

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biofilms and their exudates have been shown to resuscitate VBNC, which might open an avenue to culture VBNC in the laboratory setting (Buddhika & Seneviratne, 2019). The VBNC state of pathogenic bacteria including *Vibrio cholerae* and enteropathogenic *Escherichia coli* has been intensively studied and suggested to be the recessive cause of some human diseases (Colwell et al., 1996; Makino et al., 2000). However, problems caused by VBNC state bacteria in food industry have been neglected. Food and its surrounding environment are a complex and could be factors inducing the VBNC state of foodborne bacteria (Mougin et al., 2019; Cao et al., 2019; Chen et al., 2018). *E. coli* O157:H7 and *Salmonella typhimurium* have been demonstrated to enter into the VBNC state in refrigerated pasteurized grapefruit juice (Nicolo et al., 2011). VBNC state foodborne pathogens including *Salmonella enterica*, have been identified to be responsible for foodborne outbreaks (Asakura et al., 2002). Furthermore, acetic acid and lactic acid bacteria have been reported to enter into the VBNC state in wine and beer, and VBNC lactic acid bacterial cells have even caused beer spoilage cases (Liu, Deng, Li, et al., 2018). Thus, the VBNC state of foodborne pathogens and spoilage bacteria has been a challenge in food safety. Up to date, a number of spoilage bacteria have been shown to be capable of forming VBNC state, and cells in VBNC state are viable and maintain food spoilage capability. However, a critical concern remains as it has never been confirmed the VBNC cells cause such food spoilage. Thus, culturing based microbial detection method is still golden standard in food industry as no direct evidence showing the existence of VBNC cells in food products and future cause food spoilage or poisoning case.

In the current study, we aimed at using a novel procedure, in combination of genomic sequencing, flow cytometry and routine culturing, to confirm a beer spoilage case is caused by *Pediococcus damnosus* in the VBNC state.

2. Materials and methods

2.1. Bacterial strain and culture condition

A spoiled beer sample (lager beer, pH 4.5, ethanol ≥3.6% v/v, bitterness units 7, stored at 4 °C for 3 months after manufacturing with an expiration time of 12 months) with turbidity and undesired smell was acquired from a local brewery in 2014. Following culturable and viable cell numbers assessment, *16S rRNA* sequencing, and genomic sequencing, one *P. damnosus* strain, designated BM-PD14610, was harvested from the spoiled beer sample. During the identification process, the spoiled beer sample was maintained at 4 °C. The harvested cells were maintained as glycerol stock at −80 °C. A small amount of glycerol stock was spread onto MRS agar (Oxoid, UK) and incubated at 26 °C for 3–7 days. A single colony was transferred to 1 mL of MRS broth (Oxoid, UK) and incubated anaerobically at 26 °C, 200 rpm for 24 h to obtain logarithmic growing cells prior to further experiments.

2.2. Determination of VBNC cells

The existence of VBNC cells was determined by cell viability and culturability test. Cell viability test was performed using a Live/Dead BacLight bacterial viability kit (Molecular Probes, USA) combined with flow cytometry. Two fluorescent dyes propidium iodide (PI) and SYTO 9 were applied to indicate permeabilized and total cells, respectively. In brief, the fluorescent dyes PI and SYTO 9 were mixed in a 2:1 ratio. Washed cell samples were stained with dye mixture in the dark for 20 min. Logarithmic growing cells and heat-killed cells were served as positive controls for viable and permeabilized cells, respectively. Thus, viable and permeabilized cell numbers could be assessed by NovoCyte flow cytometer with NovoExpress 1.2.5 software (ACEA Biosciences, Inc.). With SYTO 9 (FITC) as x-axis and PI (PIPE-Texas Red) as y-axis, the cells fall into the fourth quadrant are identified to be viable cells. Culturable cells enumeration was carried out by routine MRS agar plate counting. For regular identification, 100 μL of culture were plated on MRS agar plate followed by 48 h incubation at 26 °C. For enrichment, 10 mL of sample were enriched by centrifugation at 1000×*g* for 10 min and resuspended in 100 μL of distilled water prior to MRS agar plating. The difference between viable and culturable cell numbers was designated to be VBNC cell number. When culturable cell number reached 0 (no colony appears when plating 100 μL culture on plate), all the viable cells entered into the VBNC state.

2.3. 16S rRNA and genome sequencing

To identify the bacterial species causing the spoilage of the beer sample, both culturable and viable cells in 500 mL of the spoiled beer sample were enriched by centrifugation at 1000×*g* for 10 min and adapted to DNA isolation using a Bacterial genomic DNA extraction kit (Dongsheng Biotech Co., Ltd, China) according to the instruction. The genomic DNA was used as a template to amplify *Lactobacillus 16S rRNA* gene (F: AGAGTTTGATCCTGGCTCAG, R: CTACGGCTACCTTGTTACGA) and *Pediococcus 16S rRNA* gene (F: CTACGGGAGGCAGCAAG, R: ATTACCGCGGCTGCTGG). The PCR product was purified and adapted to Sanger sequencing (IGE Biotech LTD, China). The sequence was aligned to the currently available sequences in GenBank using BLASTn.

To identify if the *P. damnosus* cells in the spoiled beer sample are from the same strain, the genomic DNA of *P. damnosus* cells from the spoiled beer sample was also sequenced by the Illumina HiSeq 2500 platform and paired-end libraries. The read qualities were examined and filtered by FastQC v.0.10.1. The filtered reads were assembled *de novo* into scaffolds through Velvet software v1.2.08 (Zerbino & Birney, 2008).

2.4. Confirmation of VBNC state

To confirm the existence of VBNC state, nonculturable cells were further adapted to propidium monoazide (PMA)-PCR assay (Liu et al., 2017a). As PMA penetrates only into permeabilized bacterial cells with compromised membrane integrity but not into live cells with intact cell membranes, PMA treatment to cultures with both viable and dead cells result in selective removal of DNA from dead cells. The positive amplification of PMA treated nonculturable cells indicates the existence of viable cells (i.e. VBNC cells). PMA was added to nonculturable cells and the mixed samples were incubated on ice in dark for 10 min. Cooled samples were exposed to halogen light (with a distance of 15 cm) for 5 min for covalent binding of PMA to DNA. PMA binding DNA was extracted using a Bacterial genomic DNA extraction kit (Dongsheng Biotech Co., Ltd, China) according to the instruction and used as a template to amplify *P. damnosus 16S rRNA* gene (F: CTACGGGAGGCAGCAAG, R: ATTACCGCGGCTGCTGG). PCR were conducted following the cycling program: an initial heating at 94 °C for 3 min, followed by 30 cycles of 94 °C for 50 s, 51 °C for 50 s, and 72 °C for 1 min, with a final 10 min extension at 72 °C. PMA-PCR were conducted in triplicate to ensure reproducibility.

2.5. Low temperature storage system

The low-temperature storage system was set up to mimic the beer storage process, a potential stress condition to *P. damnosus*. Approximately 10^7 logarithmic growing *P. damnosus* cells were inoculated and anaerobically cultured at 26 °C in 10 mL of degassed and autoclaved commercial beer. The logarithmic growing cells were harvested at 4 °C (centrifugation at 2800×*g* for 15 min) and washed twice with phosphate buffer (PBS). Then the washed cells were filtered and resuspended in 10 mL of degassed and autoclaved beer at a final density of 10^7 cells/mL and maintained at 0 °C without shaking. VBNC state determination was performed every 7 days.

2.6. Beer subculturing system

The beer subculturing system was performed to mimic the beer

processing as previously described (Deng et al., 2015). The samples preparation was performed as described in the low-temperature storage system set up. The cells were filtered and resuspended in 10 mL of degassed and autoclaved beer at a final density of 10^7 cells/mL and anaerobically cultured at 26 °C (1st generation). After 7 days incubation, cells from 10 ml 1st generation were filtered and reinoculated in fresh degassed and autoclaved beer at 26 °C (2nd generation). The interval of each subculture and VBNC state determination were 7 days.

2.7. Resuscitation strategies

The VBNC cells (approximately 10^5 cells/mL) acquired from the low temperature and beer subculturing system were subsequently used for resuscitation. Temperature upshift and chemical addition were applied for VBNC cells resuscitation, respectively. Temperature upshift was performed by initially incubating the VBNC cells in MRS broth at 10 °C for 1 h and gradually increasing the temperature by 5 °C every 1 h until reaching 35 °C. Cell culturability was determined every hour. MRS broth and agar plates with the addition of 10 μL Tween-20, 10 μL tween-80, 0.05 g vitamin C, 0.05 g vitamin B2, and 800 U/plate catalase (Sigma-Aldrich, USA) were used, respectively, in chemical addition system (Liu et al., 2018). Different temperatures were also used for the chemical addition system in MRS broth.

2.8. Beer contamination test

Approximately 10^6 of logarithmic growing, VBNC, and resuscitated *P. damnosus* cells were inoculated into 10 mL of degassed and autoclaved beer samples at room temperature, respectively. Uninoculated beer sample was used as negative control. The beer turbidity was visually observed every 7 days. Organic acids and diacetyl concentrations were determined after 30 days by reversed-phase high performance liquid chromatography (RP-HPLC) and head space gas chromatography, respectively, and quantified by the external standard method (Liu et al., 2017).

2.9. Statistical analysis

Data are presented as mean ± standard deviation of three independent biological replicates. Statistical comparisons were performed by one-way analysis of variance followed by Tukey's comparison test (XLstat software). A p-value < 0.05 was considered to be significant.

3. Results

3.1. P. damnosus cells in VBNC state cause beer spoilage

One spoiled beer sample which had been stored at 4 °C for 3 months, was subjected to routine MRS agar culturing detection to identify contaminating source, as most of the beer spoilage cases had been reported to be caused by lactic acid bacteria (LAB). No colony appeared on MRS agar plate with 1 × beer sample, suggesting the existence of VBNC state bacteria. However, less than 10 colonies appeared in the enriched beer sample, indicating the presence of small amount of culturable cells. Thus, viable cell number was assessed to determine the existence of VBNC cells. The difference between culturable (less than 10 cells/mL) and viable cell number was approximately 5×10^2 cells/mL, showing the presence of VBNC cells in the spoiled beer sample. Thus, the bacterial cells from the spoiled beer sample were collected and subjected to further identification. According to our previous studies, VBNC state *Lactobacillus harbinensis* had been determined to cause beer spoilage case (Liu, Deng, Li, et al., 2018), and some other Lactobacillus spp. strains including *L. brevis* (Liu, Deng, Soteyome, et al., 2018), *L. casei* (Liu et al., 2017c), *L. acetotolerans* (Deng et al., 2015), *L. plantarum* (Liu et al., 2017d), and *L. lindneri* (Liu et al., 2017b) were also capable of causing beer spoilage and entering into the VBNC state. *Lactobacillus 16S rRNA* gene amplification was performed on the nonculturable cells from the spoiled beer sample. Unfortunately, the bacterial cells were not *Lactobacillus* spp. Besides *Lactobacillus* spp., *Pediococcus* spp. are also one of the most common beer spoilage bacteria. *Pediococcus* spp. are generally considered to be the most undesirable contaminant (Xu et al., 2020), with *P. damnosus* responsible for 90% of *Pediococcus*-induced beer spoilage (Kaiala et al., 2018; Behr et al., 2016). Thus, *Pediococcus 16S rRNA* gene was amplified and sequenced to determine the nonculturable cells to be *P. damnosus*. In addition, the presence of VBNC cells was confirmed by PMA-PCR, verifying the beer spoilage case was caused by the co-existence of both culturable and VBNC state *P. damnosus* cells. Furthermore, genome sequencing was performed to identify the *P. damnosus* cells. Assembly and alignment results demonstrating the culturable and VBNC cells in the spoilage sample belong to the same *P. damnosus* strain (designated BM-PD14610, GenBank accession number: LTEA00000000).

3.2. VBNC state induced by stress conditions

To simulate the beer storage and processing conditions, low temperature storage and beer subculturing systems which had previously been shown to induce the VBNC state of several beer spoilage *Lactobacilli* (Deng et al., 2015; Liu et al., 2017; Liu et al., 2018) were tested on *P. damnosus* strain BM-PD14610 strain, respectively. Both strategies were capable of inducing *P. damnosus* cells entry into the VBNC state (Fig. 1). At low temperature storage, viable cell number was significantly higher than culturable cell number since day 21 (p value < 0.05), with 81.4% of the cells entered into the VBNC state. All the viable cells (4.4×10^4 cells/mL) entered into the VBNC state since day 133. In beer subculturing experiments, the ratio of VBNC cells was 86.6% after the 1st generation (day 7) and reached 100% after the 17th generation (day 119) with a cell number of 2.3×10^5 cells/mL. The shorter time for entering into the VBNC state and the higher amount of VBNC cells observed in the beer subculturing system by comparison to the low temperature storage data, indicated the cold stress was not an essential factor for the VBNC state formation of *P. damnosus* strain. Considering cold stress is a common induction condition and capable of inducing multiple *Lactobacillus* species into the VBNC state (Deng et al., 2015; Liu et al., 2017; Liu et al., 2018), it might be a facilitating factor for the VBNC state formation of *P. damnosus* strain. In addition, acetic acid concentration had identified to be the greatest effect on the formation of VBNC state of *P. acidilactici*, followed by nutritional conditions and salt concentration (Li et al., 2020). Thus, although belonging to the same species, *P. damnosus* and *P. acidilactici* entered into the VBNC state in different conditions.

3.3. Resuscitation of VBNC cells

To collect more culturable cells of *P. damnosus* strain BM-PD14610 for further study, the harvested VBNC cells were subjected to resuscitation. VBNC state has been considered a survival strategy of bacterial cells under stress conditions and partial portion of the VBNC cells was capable of resuscitating when certain stresses alleviated (Oliver, 2010). Considering the cold, oligotrophic and oxidative stress conditions that might be suffered by the *P. damnosus* cells during beer processing and cryopreservation, temperature upshift and chemical addition were tested to relief stress and resuscitate the VBNC cells. However, temperature upshift, addition of Tween-20, tween-80, vitamin C, or vitamin B2 did not enable VBNC cells resuscitation, indicating cold and oligotrophic stress were not the key conditions for the VBNC state formation of *P. damnosus* strain. With the addition of catalase, which had been reported to relieve oxidative stress and promote the recovery of nonculturable cells (Jallouli et al., 2010; Kong et al., 2014), culturable cells of *P. damnosus* strain were obtained within 3 days. High concentration of hop bitter compounds in beer had been reported to exert antibacterial effect and transmembrane redox reactions, causing intracellular

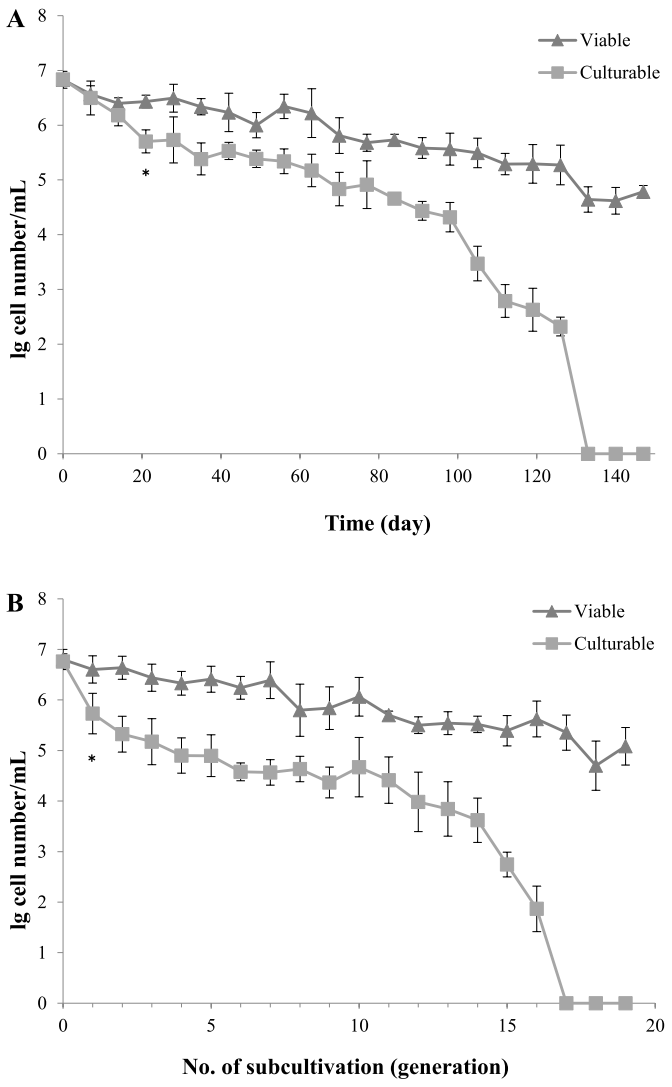

Fig. 1. Viable (▲) and culturable (■) cell numbers during VBNC inducing process in low temperature storage (A) and beer subculturing (B) systems. "*" indicates p value < 0.05.

oxidative damage (Behr & Vogel, 2010). The VBNC *P. damnosus* cells might be stressed and sensitive to oxidative stress and induce VBNC state as an adaptation to resist hop compounds encountered in beer. Thus, the antioxidant capacity of catalase may alleviate the stress posed to *P. damnosus* cells. Coupled with the induction results, oxidative stress posed by beer might play important role for *P. damnosus* to enter into the VBNC state.

3.4. Beer contamination by VBNC cells

According to the beer contamination test, logarithmic growing, VBNC, and resuscitated *P. damnosus* cells caused visually beer turbidity and undesirable smell and flavor in 7 days. Although the organic acids and diacetyl produced by VBNC cells were lower than those produced by logarithmic growing and resuscitated cells, the VBNC cells were capable of causing unbalanced acid and diacetyl contents in beer (Table 1). The results verified VBNC *P. damnosus* cells were the cause of beer spoilage case. Thus, besides *Lactobacillus* spp. (Deng et al., 2015; Liu et al., 2017; Liu et al., 2018), the detection and control of VBNC state *Pediococcus* spp. strains are also of importance in beer industry.

Table 1
Organic acids and diacetyl concentrations in *P. damnosus* spoiled beer.

State	Diacetyl (mg/L)	Lactic acid (mg/L)	Acetic acid (mg/L)
Negative control	0.03 ± 0.00 [a]	63.1 ± 13.4 [a]	117.6 ± 28.8 [a]
VBNC	0.07 ± 0.01 [b]	150.0 ± 25.2 [b]	139.3 ± 24.9 [b]
Resuscitated	0.14 ± 0.02 [c]	209.1 ± 48.5 [c]	159.4 ± 39.0 [c]
Logarithmic growing	0.16 ± 0.02 [c]	213.2 ± 33.8 [c]	179.9 ± 28.7 [c]

4. Discussion

Based on years of study in the field of VBNC state, an important concern about the food safety issues caused by bacteria in VBNC state has been raised. The issue caused by VBNC state in food industry is common, not only for spoilage microbes, but also for pathogenic microbes. However, culturing methodology is still golden standard, as people would ask, if anyone could provide direct evidence for this? Rare study could, because VBNC is nonculturable, mostly it is hard to recover the strain. Also, one important way to control food safety problem, is by accurate detection. Once bacterial cells form VBNC state, the "golden standard" culturing methodology is incapable of detecting, let alone identifying such bacterial cells. However, when we obtain a spoiled food sample (within expiration date, likely to be caused by microbes), how we can confirm it is caused by bacteria in VBNC state, or which bacterial species actually is responsible for this spoilage? Suppose within this spoiled food samples, there are different species, including strain A (both in culturable and VBNC), strain B (both in culturable and VBNC), strain C (only in VBNC), etc. A few methodologies to be used are discussed as follows: 1. If we subject the sample to culturing on an agar plate, partial cells of A and B will be found. However, it is possible that strain C is the responsible spoilage bacteria instead of A and B. 2. If we apply PMA (or other fluorescent dye) with nucleic acid amplification detection, we are able to tell the existence of viable cells, and for the species to be detected, it depends on the primers pairs we use. For example, if we use PMA-PCR on *femA* gene, positive result could tell there is viable *S. aureus* cells (Jiang et al., 2021). Same for *rfbE* gene on *E. coli* and *invA* gene on *Salmonella* (Ou et al., 2021; Zhou et al., 2020). Another way is, we can use *16S rRNA* gene, however, this could only tell there is viable bacterial cells inside the food samples, but which species still remain unknown. 3. If we use Live/Dead BacLight bacterial viability kit with flow cytometry or microscopy, it only also tells there is viable cells inside, but which species remains unclear.

In this study, we had obtained a spoiled beer samples within expiration date. At the very first, we had no idea what species, how many species and in what state of the microbes. Our approach is, firstly, we used the spoiled food sample for DNA isolation and perform genomic sequencing by Illumina/PacBio. This is the only way to confirm how many and what species are within the food sample. In the example above, via genomic sequencing, we can determine there is strain A or/and B or/and C within. An alternative is *16r RNA* sequencing, however, this can only work on bacteria than fungi, mostly can only identify to the genus level and may raise significant inaccuracy issue. In our study, we had performed genomic sequencing on the beer samples by Illumina. According to the sequencing results, only one distinctive genome as *P. damnosus* was found, which ruled out the possibility of existence of other strains. However, at this point, we were still unable to determine what state the detected microbes are in and the characteristics of the microbes (for example, are they really spoilage bacteria, are they capable of entering VBNC state and cause further food spoilage). Secondly, we had subjected the spoiled beer sample to culturing on MRS and LB agar plates, followed by CFU counting and routine bacterial identification including morphology observation, biochemistry confirmation and PCR. Thirdly, we had dyed the viable cells using Live/Dead BacLight bacterial viability kit and perform viable cell counting on flow

cytometry. Via the difference between viable cells and culturable cells, we further confirmed the approximate number of VBNC cells in the spoiled beer sample. In the example above, combining these 2 methods, it is able to determine the state of existent microbes (like strain A and/or B and/or C). Thirdly, since we had recovered one *P. damnosus* strain from MRS plate, we further subjected this isolate for genomic sequencing, to confirm the recovered strain on the plate and the one in beer sample are identical. Further investigation on this strain was performed. In addition, we had performed VBNC induction to mimic the environments of the beer and the results showed this strain is capable of entering into the VBNC state under beer subculturing and low temperature storage. At last, we had performed spoilage experiment to show this strain is capable of producing different substances to spoil beer sample.

5. Conclusion

In the current study, we used a novel procedure to confirm a beer spoilage case caused by a *Pediococcus damnosus* strain in the VBNC state. A beer spoilage case was first determined to be caused by the co-existence of culturable and VBNC state *P. damnosus* strain BM-PD14610. In addition, VBNC cells were obtained in both simulation conditions, including beer low temperature storage and beer subculturing. Furthermore, MRS agar supplemented with catalase was found to resuscitate VBNC cells, and normal, VBNC and resuscitated cells showed similar level of beer spoilage capability. This study, and the procedure we had used, will aid in further VBNC study so more evidence on the food safety problem caused by VBNC microbes will be shown. I believe this will further unveil how much of a role VBNC cells are playing in food spoilage and poisoning, as this has posed the critical issue for culturing method thus influences its accuracy.

CRediT authorship contribution statement

Zhenbo Xu: Funding acquisition, Validation. **Kan Wang:** Validation, Revision. **Ziqi Liu:** Writing – original draft, Data curation. **Thanapop Soteyome:** Methodology, Supervision. **Yang Deng:** Methodology, Supervision. **Ling Chen:** Funding acquisition, Supervision. **Gamini Seneviratne:** Validation. **Wei Hong:** Supervision, Revision. **Junyan Liu:** Conceptualization, Validation, Writing – review & editing. **Janette M. Harro:** Writing – review & editing. **Birthe V. Kjellerup:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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